

APPLICATION  
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TITLE: SPECTRAL IMAGING OF DEEP TISSUE

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## **SPECTRAL IMAGING OF DEEP TISSUE**

### **BACKGROUND**

Optical imaging of deep tissue is used to probe structures within biological specimens for laboratory research and biomedical purposes. This includes the imaging of internal organs and subdermal tissue in animals such as mice, zebrafish, or humans, and one of the goals is to learn about internal structures without surgery or other intrusive measures.

In one technique of deep tissue imaging, fluorescent probes which bind to a specific target in the specimen are imaged by exciting them with illumination light, causing them to fluoresce; the fluorescent emission is separated from the illumination light, which has a different wavelength, by barrier filters and then is detected using a very sensitive camera such as a cooled CCD detector. In other techniques, the specimen is infected with agents that cause it to produce material that is inherently fluorescent, with the most common example being green fluorescent protein (GFP). Further techniques involve use of quantum dots as luminous probes.

As used herein, compounds such as fluorescent probes, GFP, or quantum dots, as well as related compounds or others used for similar purposes, are all termed the target compounds of a measurement.

The signals produced in such experiments are typically weak. In general, robust detection of the weak levels of light emitted from the deep structures is beneficial because it provides earlier, or more reliable, detection of the structures being studied. Also, it may enable detection of lower levels of the target compound. Accordingly, techniques or apparatus used for deep tissue imaging are valued if they offer a low detection threshold.

### **SUMMARY**

The present invention features a method and apparatus to improve detection of target compounds such as GFP, quantum dots, and fluorescent probes used for deep tissue imaging in a variety of biological samples. Moreover, the method and apparatus are generally compatible with use of such compounds, without requiring extreme sensitivity from the imaging detector.

The method and apparatus use spectral imaging to distinguish between undesired autofluorescence signals arising from various sites in the specimen, and the desired signal from the target compound. It has been discovered that the spectral information thus obtained provides improved detection sensitivity and can be used for deep tissue imaging, even though there is typically significant optical loss in the spectral selection elements of the system.

In one embodiment, images are taken while viewing the emission light from the specimen at a sequence of wavelengths, to develop an image cube with two spatial dimensions and a spectrum at each point. By determining the difference in spectral properties between the desired target compound emission and the unwanted autofluorescence emission, the overall signal is decomposed into components and the detection levels for the desired compound emission are greatly improved.

Other embodiments include measurements based on brightness ratios at several selected wavelengths or wavelength bands; measurements based on principal component analysis; and measurements based on neural networks, and on fuzzy logic.

In either case, the method and apparatus use spectral information to distinguish the desired signal emitted by the target compound from the unwanted autofluorescence signal, and thus to improve the measurement integrity and sensitivity.

Various aspects and features of the invention will now be summarized.

In general, in one aspect, the invention features a method including: (i) illuminating a sample to cause the sample to emit radiation, wherein the sample includes deep tissue supporting a target compound, and wherein the emitted radiation includes emission from the target compound and emission from one or more other components in the sample; (ii) spectrally filtering the emitted radiation with each of a plurality of different spectral weighting functions; (iii) storing an image of the spectrally filtered radiation for each of the spectral weighting functions; and (iv) processing the stored images to construct a deep tissue image of the sample in which signal from the other compounds is reduced relative to signal from the target compound.

In general, in another aspect, the invention features a method including: (i) providing a plurality of images of spectrally filtered radiation emitted from a sample in response to an illumination, wherein the sample includes deep tissue supporting a target compound, wherein the emitted radiation includes emission from the target compound and emission from one or

more other components in the sample, and wherein each image corresponds to a different spectral weighting function; and (ii) processing the images of the spectrally filtered radiation to construct a deep tissue image of the sample in which signal from the other compounds is reduced relative to signal from the target compound.

5           In general, in yet another aspect, the invention features an apparatus including a computer readable medium which stores a program that causes a processor to: (i) receive a plurality of images of spectrally filtered radiation emitted from a sample in response to an illumination, wherein the sample includes deep tissue supporting a target compound, wherein the emitted radiation includes emission from the target compound and emission from one or  
10       more other components in the sample, and wherein each image corresponds to a different spectral weighting function; and (ii) process the images of the spectrally filtered radiation to construct a deep tissue image of the sample in which signal from the other compounds is reduced relative to signal from the target compound.

          In general, in yet another aspect, the invention features an apparatus comprising: (i) a  
15       sample holder configured to hold a sample including deep tissue, wherein the deep tissue supports a target compound; (ii) an illumination source configured to illuminate the sample to cause it to emit radiation, wherein the emitted radiation includes emission from the target compound and emission from one or more other components in the sample; (iii) an imaging system configured to image the emitted radiation to a detector; (iv) a tunable spectral filter  
20       configured to spectrally filter the emitted radiation with each of a plurality of different spectral weighting functions; (v) a detector configured to store an image of the spectrally filtered radiation for each of the spectral weighting functions; and (vi) a electronic processor configured to process the store images to construct a deep tissue image of the sample in which signal from the other compounds is reduced relative to signal from the target  
25       compound. For example, the sample holder may configured to hold an animal, such as a mammel, like a mouse, rabbit, or human. Also, for example, the imaging system may have a demagnification greater than or equal to 1, and, for example, the imaging system may be configured to image a field of view having a diagonal dimension greater than about 2 cm onto the detector.

30           Embodiments of the various aspects of the invention described above may include any of the following features.

The sample including the deep tissue may be a living organism, such as an animal or a mammal. For example, the animal may include a mouse, a rabbit, a zebrafish, or a human. Also, the deep tissue may be an internal organ of the living organism, and

the deep tissue may lie within about 2 mm or more of the living organism.

5 The deep tissue may be subdermal tissue.

The emission from the other components of the sample may include autofluorescence from tissue overlying the deep tissue.

The emission from the other components of the sample may include autofluorescence from one or more layers of tissue in the sample different from a layer of tissue including the  
10 deep tissue.

The target compound may be any of, for example, a fluorescent probe bound to at least a portion of the deep tissue, a quantum dot bound to at least a portion of the deep tissue, a green fluorescent protein (GFP) bound to at least a portion of the deep tissue, a yellow fluorescent protein (YFP) bound to at least a portion of the deep tissue, and a red fluorescent  
15 protein (RFP) bound to at least a portion of the deep tissue.

The emission from the target compound may be fluorescence.

At least some of the spectral weighting functions may correspond to particular wavelength bands. For example, all of the spectral weighting functions correspond to particular wavelength bands. Alternatively, at least some of the spectral weighting functions  
20 may correspond to sinusoidal weightings of multiple wavelength bands.

The spectral filtering may include using any of a liquid-crystal, tunable optical filter, an interferometric optical filter, and a filter wheel containing a plurality of band pass filters.

Each stored image may include an intensity value for each of multiple pixels.

Processing the stored images may include constructing the deep tissue image based  
25 on a weighted superposition of signals in the stored images.

Processing the recorded images may include constructing the deep tissue image based on the recorded images and at least one emission spectrum for the other components in the sample. For example, constructing the deep tissue image may include calculating a remainder spectrum for each pixel in the set of stored images based on the at least one  
30 emission spectrum for the other components.

Similarly, processing the recorded images may include constructing the deep tissue image based on the recorded images and an emission spectrum for the target compound. For example, constructing the deep tissue image may include calculating a remainder spectrum for each pixel in the set of stored images based on the emission spectrum for the target compound.

Also, processing the recorded images may include constructing the deep tissue image based on the recorded images, at least one emission spectrum for the other components in the sample, and an emission spectrum for the target compound. For example, constructing the deep tissue image may include solving at least one component of a matrix equation in which one matrix is based on the stored images, and another matrix is based on the emission spectra.

The deep tissue may support multiple target compounds and processing the stored images may include constructing a deep tissue image for each of the target compounds. For example, processing the recorded images may include constructing the deep tissue images based on the recorded images and emission spectra for the target compounds. Furthermore, processing the recorded images may include constructing the deep tissue images based on the recorded images, the emission spectra for the target compounds, and at least one emission spectrum for the other components in the sample.

The plurality of the different spectral weighting functions may include four or more spectral weighting functions.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features, objects, and advantages of the invention will be apparent from the following detailed description.

## DESCRIPTION OF DRAWINGS

The invention will now be further described merely by way of example with reference to the accompanying drawings.

Figure 1 is an image of a mouse which has been injected with a fluorescent probe, imaged at a single spectral band of  $\lambda = 530$  nm.

Figure 2 is a graph of the emission spectrum of autofluorescence and that of the target compound for the sample of Figure 1, with the light-colored line showing the spectrum for the autofluorescence and the dark-colored line showing the spectrum for the target compound.

Figure 3 is an image of the target compound emissions from the mouse of Figure 1, with the autofluorescence signal removed using spectral techniques in accordance with the present invention.

Figure 4 is an image of the autofluorescence emissions from the mouse of Figure 1, with the target compound emissions separated using spectral techniques in accordance with the present invention.

Figure 5 is a flow-chart of one embodiment of the present invention, based on acquisition of a spectral cube and subsequent analysis by linear unmixing.

Figure 6 is graph of the spectral properties of each spectral band in the embodiment of Figure 5.

Figure 7 is a flow-chart of another embodiment which incorporates PCA to estimate the spectra of the autofluorescence and the target compound(s).

Figure 8 is a flow-chart of yet another embodiment in which the spectral filtering is performed interferometrically.

Figure 9 is a graph of the spectral properties of the spectral weightings used in the embodiment of Figure 8.

Figure 10 is a schematic diagram of a spectral imaging system.

Like reference symbols in the various drawings indicate like elements.

## DETAILED DESCRIPTION

The invention features a method and apparatus for reducing the detection level of a target compound in deep tissue through spectral discrimination. An important aspect is that a beneficial result is obtained despite the low light levels involved.

A schematic diagram of a spectral imaging system 100 for imaging deep tissue is shown in Figure 10. System 100 includes a sample holder 110 suitable for holding a

specimen 112 having deep tissue. For example, the specimen may be a living organism, such as an animal or mammel. A target compound is bound to selected portions of deep tissue in the specimen. An illuminator 120 (e.g., a metal halide lamp or other lamp, a laser, an light emitting diode array, or any other source of electromagnetic radiation) directs excitation light 122 to the specimen to excite emission (e.g., fluorescence) from the target compound in the deep tissue. Typically, the excitation light will also cause the autofluorescence from the other components in the specimen. Therefore, the electromagnetic radiation 130 emitted from the specimen includes emission from the target compound as well as autofluorescence. Emitted radiation 130 is collected by imaging system 140 and imaged onto a camera 150.

Because system 100 is designed for imaging deep tissue in relatively large specimens (e.g., living organisms), the imaging system typical provides a demagnification of one or more, or even 2 or more. That is, the image on the camera is the same size or smaller than the object field of view for the imaging system. Also, the object field of view for the imaging system is typically greater than about 2 cm (or greater than about 3 cm) along a diagonal dimension.

Furthermore, although Figure 10 shows emitted radiation 130 as being collected from an opposite side of the specimen relative to excitation light 122, in other embodiments, the emitted radiation can be collected from the same side as, or at an angle to, that illuminated by the excitation light. Moreover, illumination may be provided from multiple sides of the specimen.

Positioned between the specimen and camera 150 is a tunable optical filter 160 (e.g., a liquid crystal, tunable optical filter, an interferometric optical filter, or a motorized filter wheel). Optical filter 160 spectrally filters emitted radiation 130 according to each of a plurality of spectral weighting functions (for example, four or more spectral weighting functions). The spectral weighting functions may correspond to specific wavelength bands, or may be more complicated functions such as a sinusoid distribution of pass bands. Camera 150 records images of the spectral filtered emitted radiation 170 for each of the different spectral weighting functions, and sends the image data to a computer 180 for analysis. As described in greater detail below, the computer processes the image data based on the different spectral weighting functions, and one or more emission spectra corresponding to pure target compound, pure autofluorescence of one or more other components of the



specimen, or both, to construct a deep tissue image that suppresses the autofluorescence signal to reveal the target compound.

In what follows, we describe the context for the spectral imaging of the deep tissue, a specific example of deep tissue imaging, and spectral unmixing techniques for constructing the deep tissue image.

It is a hallmark of imaging structures in deep-tissue samples via target compounds that the optical signals are relatively weak. Accordingly, many practitioners place prime importance on the properties of the detector, and on the efficiency of all elements in the optical path, such as the lenses and the blocking filter used to block the excitation light from reaching the detector. Yet while the present art of CCD detectors and the like is suitable for detecting low light level signals, it does not adequately address the problem of discriminating between light emitted by the target compound, and light from other sources such as autofluorescence. Thus one's detection level in practice may be set by the level of confounding light arising from sites elsewhere within the specimen, rather than considerations such as readout noise in one's detector, or the light gathering power of the objective.

Put more precisely, one's detection limit can be seen as the greater of one's detector noise or the confounding signal flux which is presented to the detector; expressed in either case as the equivalent concentration of target compound in the specimen to produce light of that signal level at the detector.

Unless the light emitted by the target compound dominates over all other sources in the specimen, one is often limited by the confounding signal rather than by one's detection apparatus. Some level of autofluorescence is inherent in biological samples when they are illuminated with light of visible range, especially when the light is green (550 nm) or shorter. So despite the use of optimized target compounds, autofluorescence arising at or near the specimen surface can often set the detection limit.

Further, emission from target compounds within deep tissue can be attenuated by scattering or absorption as it travels from the site of emission to the surface of the specimen. The signal level reaching the imaging system is thus reduced, while light that is generated at the surface layer of the specimen is not similarly attenuated. The details of this effect depend

on the geometry of the sample specimen relative to the collection optics, as well as the optical properties of the sample.

5 Likewise, the illumination light may be attenuated or scattered as it travels from the source of illumination through the surface layers of the specimen on its way to the structure being imaged. The excitation signal reaching the target site is reduced, while the signal developed at the surface of the specimen is not similarly attenuated. The details of this depend on the geometry of the illumination and collection optics, as well as on the optical properties of the sample specimen.

10 These considerations can exacerbate the effect of autofluorescence by increasing the relative contribution of autofluorescence emission to the detected signal, compared with emission from the target compound.

The magnitude of the problem is illustrated by Figure 1, which is an image of the fluorescent emission from a mouse. The mouse was illuminated with light of approximately 480 nm, and the emission light was filtered by a 25 nm bandpass filter centered at 530 nm.  
15 There is a tumor in the lung of the mouse which expresses the green fluorescent protein (GFP). Yet the tumor is not easily distinguishable in the image due to an equal or greater signal from generalized autofluorescence, apparently developed in the dermal layers of the mouse. As a result, while the signal level at any point in the image is easily quantified, the presence of target compound, and thus the tumor, cannot be confirmed due to high levels of background autofluorescence which sets an equal or higher detection threshold than the  
20 target compound involved.

Autofluorescence is also variable from specimen to specimen and can be unpredictable. Thus if an absolute flux level is used to make assessments about the target compound, one can obtain false positive readings. Variability can arise from factors such as  
25 mold or disease on the skin of the specimen. These are typically not uniform across the specimen. So if one seeks to detect the presence of a target compound by comparing local signal levels in a given region against the mean level for the specimen, results are also not reliable.

It is possible in some cases to reduce autofluorescence by choice of the illumination wavelength. Generally the use of longer wavelengths for illumination is beneficial, as is  
30 known in the art, since they typically generate less autofluorescence. Also, it can be

beneficial to choose a target compound whose emission light occurs at a different wavelength range from the autofluorescence of the specimen. Yet it is not possible to choose wavelengths that is free of crosstalk, as shown by Figure 2. The emission spectra of the target compound, GFP (shown by the dark-colored line), and of the autofluorescence (shown by the light-colored line), are completely overlapping. At any wavelength where the target has substantial emission, the autofluorescence is also strong, so autofluorescence cannot be eliminated by use of a fixed optical filter or something similar. Nor does a color camera discriminate between two such similar green spectra.

Yet as Figure 2 indicates, the spectra of GFP and autofluorescence emissions are nonetheless different. Thus, in accordance with the invention, a spectral imaging approach can distinguish the two and eliminate or greatly reduce the contribution of the latter signal.

In a first embodiment of the invention, this is achieved with conventional apparatus to illuminate the specimen and to block the illumination light from entering the detector. This can be done using an illuminator such as the LT-9500 MSYS from Lighttools Research (Encinitas, CA) together with a longpass optical filter that transmits substantially all light  $\lambda > 510$  nm, placed in the path of the objective.

The spectral imaging detector consists of a QImaging 1300C digital cooled CCD camera (Roper Scientific, Trenton NJ) with a 55 mm F/2.8 Nikkor macro lens (Nikon USA, Melville NY), to which a VARISPEC tunable optical filter (CRI Inc, Woburn MA) is coupled with a mounting adaptor. The VARISPEC filter is a computer-controlled optical filter with 25 nm bandpass and tunable center wavelength. These are connected to an IBM Thinkpad computer which controls the image acquisition and performs the data analysis. Communication is via an IEEE-1394 interface to the camera, and an RS-232 interface to the VARISPEC filter.

The VARISPEC filter uses nematic liquid crystal variable retarder elements to construct a tunable Lyot filter. The variable retarders are placed in optical series with fixed waveplates of quartz or other material, to produce a retardance that is well-known and electrically adjustable. Linear polarizers between successive stages of the filter act to block unwanted orders so only a single peak is transmitted, and out-of-band leakage can be reduced to 0.01% if desired. By choice of the retarder thicknesses, one may obtain bandwidths

ranging from 0.1 nm to 50 nm or more. Tuning action is rapid (< 50 ms) and there is no image shift from tuning, which is valuable for imaging applications.

The mouse was imaged by taking a sequence of images while the center wavelength of the VARISPEC filter was tuned from 500 nm to 650 nm. The result is an image cube, with a full two-dimensional image of the sample, and a full spectrum at every point in the image. The exact spectrum recorded at a given point depends on the amount of GFP and autofluorescence, and on the two spectra, as:

$$S(x, y, \lambda) = a(x, y) * F(\lambda) + b(x, y) * G(\lambda) \quad [1],$$

where (x, y) indices are used to denote a given pixel location in the image, the asterick "\*" denotes multiplication,  $\lambda$  is used to denote a given wavelength of emission or detection, and

$S(x, y, \lambda)$  denotes the net signal for a given location and wavelength,

$F(\lambda)$  denotes the emission spectrum of autofluorescence,

$G(\lambda)$  denotes the emission spectrum of GFP,

$a(x, y)$  indicates the abundance of autofluorescence at a given (x, y) location, and

$b(x, y)$  indicates the abundance of GFP at a given (x, y) location.

Equation [1] states that the net signal from a given location is the sum of two contributions, weighted by the relative amount of autofluorescence and GFP present. It is easier to see if one writes the above equation for a single pixel:

$$S(\lambda) = a F(\lambda) + b G(\lambda) \quad [2].$$

F and G may be termed the spectral eigenstates for the system, which are combined in various amounts according to the amount of autofluorescence and GFP emission, to produce an observed spectrum S.

Now if the emission spectra of the autofluorescence and of the GFP are known (or can be deduced, as described below), one may invert equation [2] by linear algebra to solve

for a and b, provided that the spectrum S has at least two elements in it; i.e. that one has data for at least two emission wavelengths  $\lambda$ . Then we can write

$$A = E^{-1} S \quad [3],$$

5

where

A is a column vector with components a and b, and

E is the matrix whose columns are the spectral eigenstates, namely [F G].

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Using equation [3] one can take the observed spectrum and calculate the abundance of the autofluorescence and of the GFP sources. This process may be repeated for each pixel in the image, to produce an image of GFP that is free of contributions from autofluorescence. As a result, the detection level is greatly enhanced.

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Note that the matrix E need only be inverted once for a given set of autofluorescence and target compound spectra, so the calculation of abundances is not burdensome and can be readily done in nearly real-time by a personal computer.

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The results of this process are shown in Figures 3 and 4, which present the abundance images for GFP and autofluorescence, respectively. As Figure 3 shows, it is easy to detect the GFP once it is separated from the autofluorescence. The degree of improvement in the GFP image is striking. Also, one can see that the autofluorescence image is smooth and unaffected in the region where GFP is present, which is consistent with the fact that the presence of GFP in a deep tissue structure should not alter the amount of autofluorescence emission from the overlying dermal regions.

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Overall, the measurement and analysis process is shown as a block diagram in Figure 5. The specimen is prepared and illuminated (step 505) and the spectral bands to be acquired determined (step 510). Then the spectral filter is set to transmit the spectral weighting function  $I_i$ , for example, a particular wavelength band (step 515), and an image corresponding to that spectral weighting function is acquired (step 520). The spectral filter is then set to the next spectral weighting function and the corresponding image acquired until all bands have been acquired (steps 525 and 530). The spectra for the target compound and the autofluorescence is then provided or otherwise determined (step 535). Based on the

30

spectra, the matrix E is generated and its inverse determined (step 540). For each image pixel, the spectra defined by the series of acquired images is then multiplied by the inverse matrix of E (step 545) to generate an abundance image of the target compound(s), i.e., the deep tissue image (step 550).

5           In this example, the invention permitted observation of structures in tissue lying ~2 mm within a living organism, where the overlying dermis is at least 300 microns thick and has significant autofluorescence. The invention has also been used to image structures at differing depths in other specimens, including non-mammalian specimens such as zebrafish. In the latter, the specimen is physically thinner, but once again there is the problem of  
10 autofluorescence arising from other layers in the specimen, which confounds the detection of target compounds in the interior of the specimen. While there are optical techniques for depth sectioning, such as confocal microscopy, the present invention provides a simple and practical alternative.

          Nothing about this invention prevents one from viewing multiple target compounds  
15 per specimen. If we denote the number of spectral settings as n, matrix E becomes an n x m matrix instead of an n x 2 matrix used in the above example. So, one can use the invention to remove autofluorescence from a sample which contains two target compounds; or to remove two types of autofluorescence from a sample with one or more target compounds. In any case, the result is the isolation of the target compound(s) from the autofluorescence, and the  
20 ability to quantify one or all of these components.

          The limit to the number of compounds that can be isolated, and to the signal to noise generally, is given by the shot noise levels and the degree of spectral distinction between the emission spectra of the species being distinguished (including autofluorescence). One can describe the degree of correlation between two spectra by an angle  $\theta$ , defined by  
25

$$\theta = \arccos [(S_1 \cdot S_2) / (|S_1| |S_2|)] \quad [4].$$

          Sets of spectra for which  $\theta$  is small for two members are not as easily separated into their components. Physically, the reason for this is easily understood: if two spectra are only  
30 marginally different, it is harder to determine which species was present, and noise can easily change one's estimate of relative abundances. Criteria such as  $\theta$  can be used to help decide

what spectral bands are appropriate for a measurement, and one may try and select bands that yield a large  $\theta$  whenever possible. Or, one may make an empirical study of what bands yield the best separation, by trial and error. It can be helpful to include more bands than would appear necessary from mathematical analysis alone, in order to reduce sensitivity to slight spectral shifts from the expected shapes, as may occur due to variation between specimens and the like.

It is worth considering the optical efficiency of the measurement apparatus in the above embodiment, to understand where the inventive improvement comes from. First, the lens used was an F/2.8 type instead of an F/1.2 or F/1.8 which is more typical for this work, and this choice results in 2.4 – 5.4x less light collection. Next, the VARISPEC filter has a transmission of approximately 25 percent, and collects over a 25 nm range, in contrast to a typical interference filter which has a transmission of 80 percent and collects over a 40 nm range. This further reduces the sensitivity by a factor of 5.1x compared to equipment that might be used for this work, for an overall *reduction* in light flux of 12.3x – 27.8x compared to the best practice alternatives of the art.

The CCD camera is cooled 25° below ambient to approximately 0°C, which is typical for an ordinary CCD sensor, unlike the sensors used in imaging stations such as the ChemiPro system from Roper Scientific (Trenton, NJ), which is cooled with liquid nitrogen to attain temperatures 100° below ambient or lower.

As this suggests, the effectiveness of this technique does not arise from extreme efficiency in the gathering or collection of light; rather it comes from using spectral selectivity to identify and remove the effects of background fluorescence.

Turning now to the question of how the spectra F and G are determined, any method may be used which yields an adequate estimate of the spectra involved. For some target compounds, there is a known spectrum for the material from published references.

Alternatively, with a spectral imaging station as is used in the invention, one may obtain the spectrum directly by placing a sample containing a sufficient concentration of the target compound in front of the imager and taking its spectrum. Conversely, it is often possible to image a region of the specimen where one has *a priori* knowledge that there is no target compound in that region, and in this way one can obtain an estimate of that component.

Various data analysis techniques can be used in this process, such as principal component analysis (PCA), which identifies the most orthogonal spectral eigenvectors from an image cube, and yields score images showing the weighting of each eigenvector throughout the image. If PCA analysis is performed on an image that contains contributions  
 5 from the target compound(s) and from the background autofluorescence, the vectors from PCA can be used to develop estimates of the spectra involved.

This may be done in combination with other mathematical processing, and there are other known techniques for identifying low-dimensionality spectral vectors, such as projection pursuit, a technique described in L. Jimenez and D. Landgrebe, "Hyperspectral  
 10 Data Analysis and Feature Reduction Via Projection Pursuit", *IEEE Transactions on Geoscience and Remote Sensing*. Vol. 37, No. 6, pp. 2653-2667, November 1999.

An embodiment that incorporates PCA is shown in Figure 7 in block-diagram form. The block diagram is similar to that of Figure 5, except that step 535 is replaced with performing a PCA analysis (step 735) and determining eigenvectors corresponding to the  
 15 autofluorescence and target compound(s) (step 737) for use in the matrix E in step 540.

Whatever method is used in the determination, the goal is to derive spectra corresponding to the species sought, whether the determination is made by a direct measurement of relatively pure samples of the target compound and the autofluorescence, or a mathematical analysis of spectra that may contain mixtures of both components, so long as  
 20 adequate spectral estimates are obtained.

While the embodiments discussed above use spectral estimates for both the autofluorescence emissions and target compound emissions, this is not essential. In some cases, one may seek to reduce the autofluorescence without having *a priori* knowledge of the target compound spectrum. This can be done by looking at the signal in a given pixel, and  
 25 subtracting from it the maximum amount of autofluorescence while leaving the remaining signal that is positive definite in all spectral channels. That is, one defines a so-called remainder spectrum  $R_a(\lambda)$  for each pixel:

$$R_a(\lambda) = S(\lambda) - a F(\lambda) \quad [5a],$$

30 and then selects the largest value of parameter  $a$  consistent with  $R_a(\lambda)$  having a non-negative value in every spectral channel. The resulting spectrum  $R_a(\lambda)$  is then used as the sample



spectrum, expunged of autofluorescence. One may also make the determination of a based not on strict non-negative criterion listed above, but on some related criteria that incorporates a small negative distribution, to account for considerations such as shot noise or detector noise.

5           Alternatively, one may seek to determine the distribution of the target compound by a similar method when its emission spectrum is known, but the autofluorescence spectrum is not, by seeking to subtract off from  $S(\lambda)$  the maximum amount of target emission  $G(\lambda)$  consistent with a positive remainder, and then reporting the amount that was subtracted at each pixel as the image of the target compound. In this case, the remainder spectrum  $R_b(\lambda)$  for each pixel is given by:

$$R_b(\lambda) = S(\lambda) - bG(\lambda) \quad [5b],$$

15           where one selects the largest value of parameter  $b$  consistent with  $R_b(\lambda)$  having a non-negative value in every spectral channel.

          Furthermore, the technique described above in connection with Equations 5a and 5b can be expanded to cases where the spectra for one or more additional components of the sample are known, and one wants to remove their contribution to the signal. In such cases the remainder spectrum is rewritten to subtract a contribution of each such component from the observed signal based on the additional spectra and consistent with a positive remainder in each spectral channel.

          Another preferred embodiment uses the same apparatus to illuminate and view the specimen, except that the specimen is a zebrafish in an aqueous sample stage.

25           Yet another alternative uses the same apparatus to view a mouse that has been transfected to express either the yellow fluorescent protein (YFP) or the red fluorescent protein (RFP), or both, and produces images of the target compound(s) after removal of the autofluorescence signal. There are also mutant strains developed which may also be used. Any of these may be combined with the GFP when that produces useful results.

30           Further alternative embodiments view mice containing target compounds based on quantum dots, and incorporate an illuminator and filter set optimized for the quantum dot species involved.

An embodiment operating in the infrared range 600 – 1100 nm may also be constructed using a near-infrared VARISPEC filter such as the model VIS-NIR2-10-20HC.

In an alternative embodiment, the VariSpec filter is replaced with a motorized filter wheel containing a plurality of bandpass filters. Yet another embodiment uses a split-image  
5 system from Optical Insights (Tucson, AZ) to view the specimen in four spectral bands at once, albeit with lower spatial resolution. The bands are chosen to give a spectrum that distinguishes between the target compound and background autofluorescence, i.e. to have  $\cos \theta$  that is significantly different from 1, preferably 0.8 or less.

It is not necessary that the images used for the spectral analysis be acquired with  
10 bandpass filters, only that the spectral weightings of the various images be different. For example, one could use an interferometer to acquire the spectral information, as shown in Figure 8 in block-diagram form. The spectral response of the interferometer is shown in Figure 9 for some selected values of path difference  $Z$ . Images thus obtained can be used for practicing the invention, either directly or after transforming from interferograms to spectra.  
15 The suitability of using the interferograms can be checked by looking at how well they distinguish between the species involved, which can be determined by measures such as  $\cos \theta$  or by experimental study.

The block diagram of Figure 8 is similar to that of Figure 5 except that: steps 515, 520, 525, and 530 are replaced with corresponding steps 815, 820, 825, and 830, which use a  
20 interferogram as the spectral weighting function rather than particular spectral bands; there is an optional step 832, which describe Fourier transforming the spectrally filtered images to generate a spectral cube; and step 535 is replaced with step 835 determines spectra or interferogram weightings for the target compound and the autofluorescence consistent with the form of the acquired data (and optional Fourier transform) for use in generating the  
25 matrix E.

The interferometer can be a mechanical type such as a Sagnac design, or it can be a birefringent interferometer as described in U.S. Patent 6,421,131, "Birefringent  
interferometer". The latter uses fixed retarder elements such as quartz plates, together with  
30 switching apparatus, to make the retarders add or cancel one another, so that using these elements, along with variable retarder elements, one can produce any desired retardance within a wide range. When polarized light encounters this assembly, its polarization state is

changed in a manner that depends on the wavelength of light, and this can be detected at an exit analyzer polarizer. The spectral response at any particular setting of the interferometer is a sinusoid in  $1/\lambda$ , after allowing for dispersion. By taking a sequence of readings at known retardance values, and performing a fourier transform, the spectrum of the light can be  
5 determined. Such apparatus can be used in imaging systems to obtain a spectrum at every point in an image, or simply to obtain a set of images with various sinusoidal spectral response functions in the members of the set.

Indeed, any spectral imaging apparatus can be used provided that it yields adequate spectral information to distinguish emission by the target compound from background  
10 autofluorescence.

The spectral analysis and construction of the deep tissue image can be implemented in hardware or software, or a combination of both. The electronic processing can be implemented in computer programs using standard programming techniques following the methods described herein. Program code is applied to input data to perform the spectral  
15 unmixing functions described herein and generate output information such as the deep tissue image. The output information is applied to one or more output devices such as a display monitor.

Each program is preferably implemented in a high level procedural or object oriented programming language to communicate with a computer system. However, the programs  
20 can be implemented in assembly or machine language, if desired. In any case, the language can be a compiled or interpreted language. Moreover, the program can run on dedicated integrated circuits preprogrammed for that purpose.

Each such computer program is preferably stored on a storage medium or device (e.g., CD-ROM or magnetic diskette) readable by a general or special purpose programmable  
25 computer, for configuring and operating the computer when the storage media or device is read by the computer to perform the procedures described herein. The computer program can also reside in cache or main memory during program execution. For example, computer 180 in Figure 10 may includes a processor, an input/output control card, a user interface, such as a keyboard and monitor, and a memory. A program stored on a computer-readable  
30 medium is stored in the computer memory, and when executed, the program causes the processor to carry out the steps of analyzing the spectrally filtered images.

While certain embodiments have been described, alternatives may be used that achieve the same end without deviating from the spirit of the invention, according to the requirements of the task at hand and normal design considerations. It is explicitly intended that this invention can be combined with the arts of instrument design, multispectral data  
5 analysis and image analysis, and optical system design, as appropriate for a given use.

Additional aspects, features, and advantages are within the scope of the following claims.